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Involvement of HIF-1α in MLCK-dependent Endothelial Barrier Dysfunction in Hypoxia

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Key Words

Hypoxia-inducible factor-1α • Myosin light chain kinase • Endothelial cell • Barrier dysfunction • Hypoxia

Abstract

The mechanisms of endothelial barrier dysfunction induced by hypoxia are incompletely understood. Hypoxia-inducible factor-1 alpha (HIF-1α) is a key transcription factor partially responsible for hypoxia-related responses, but its role in regulation of hypoxia-induced endothelial barrier dysfunction is unclear. The aim of this study was to determine the molecular mechanism by which HIF-1α regulates endothelial barrier function during hypoxia. Endothelial cell monolayers exposed to normoxia or hypoxia were used for physiological, morphological, and biochemical analyses. The results showed that hypoxia disrupts endothelial barrier function by upregulating protein expression of myosin light chain (MLC) kinase (MLCK) and MLC phosphorylation. Hypoxia also induces HIF-1 activation by increasing HIF-1α expression, nuclear accumulation, DNA binding activity and target gene expression of HIF-1 in endothelial cells. Knockdown of HIF-1α attenuates endothelial barrier dysfunction and the increased MLCK protein expression induced by hypoxia. Inhibiting the transcription activity of HIF-1 by overexpressing factor inhibiting HIF-1(FIH) prevents the increased MLC phosphorylation and also attenuates endothelial barrier dysfunction in hypoxia. The results suggest that HIF-1α is involved in the MLCK-dependent endothelial barrier dysfunction induced by hypoxia.

Introduction

Maintenance of the semi-permeable endothelial barrier is particularly important in controlling the exchange of macromolecules and fluid between the blood and interstitial space. Changes in morphology and function of tightly connected endothelial cells lead to barrier dysfunction and microvascular leakage, resulting in tissue edema [1-3]. Transmembrane junctions and junction proteins are critical structures maintaining barrier function and preventing microvascular leakage [1-4], whereas actomyosin interaction generates contractile forces that tend to pull cells apart. A disruption of the balance between

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those adhesive and contractile forces results in endothelial barrier dysfunction. It is well established that the phosphorylation of myosin light chain (MLC) by activated myosin light chain kinase (MLCK) regulates the interaction of myosin and actin, thereby altering contractile forces and subsequently endothelial permeability [1, 5, 6].

Pathological conditions such as hypoxia and ischemia are known to damage the endothelial barrier, resulting in increased microvascular permeability and the development of vasogenic tissue edema. However, the mechanism by which hypoxia disrupts endothelial barrier function is not completely understood. Hypoxia has a comprehensive impact on many essential cellular processes [7], in which many transcription factors are involved. Among these transcription factors, the hypoxia-inducible factor-1 (HIF-1) has been regarded as the master regulator in hypoxia responses [8-12].

HIF-1 is a heterodimer complex composed of α- and β-subunits (HIF-1α and HIF-1β) [13]. HIF-1β is expressed constitutively, while HIF-1α protein levels are tightly regulated. In normoxia, HIF-1α is rapidly targeted for ubiquitin-mediated proteasomal degradation after binding to von Hippel-Lindau (VHL) protein. HIF-1α hydroxylation, mediated by prolyl hydroxylases (PDH), is necessary for the association of HIF-1α and VHL proteins [14]. In hypoxia, PDH activity is decreased, leading to the stabilization and accumulation of HIF-1α. Additionally, hypoxia exposure induces HIF-1α expression. When activated, HIF-1 induces the expression of an array of genes pivotal to hypoxia survival by binding to consensus promoter sequences [13, 15].

It has been shown that some HIF-1 target genes, such as vascular endothelial growth factor (VEGF) and nitric oxide synthase, disrupt endothelial barrier function [16]. Endothelial barrier dysfunction associated with VEGF is accompanied by the rearrangement of zonular occludens-1 (ZO-1) and claudin-1, two tight junction (TJ) proteins [17]. A recent study has demonstrated that VEGF alone down-regulated ZO-1 mRNA and protein levels and caused increased permeability in endothelial cells. Additionally, YC-1, a HIF-1α inhibitor, was shown to protect against hypoxia-induced endothelial barrier dysfunction and ZO-1 disorganization by inhibiting HIF-1α accumulation [18]. Thus, we hypothesized that HIF-1α regulates endothelial barrier function in hypoxia.

In this study, we sought to define the role of HIF-1α in regulating endothelial barrier function after hypoxia. The data show that hypoxia causes barrier dysfunction in cultured endothelial monolayers, which is MLCK-dependent. Our present data, for the first time, also demonstrate that HIF-1α is involved in the MLCK-dependent endothelial barrier dysfunction induced by hypoxia.

Materials and Methods

Cell cultures

Human pulmonary vein endothelial cell line VE was obtained from KeyGEN Technologies (Nanjing, Jiangsu, China) and grown in RPMI 1640 medium (Invitrogen, CA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100μg/ml streptomycin. VE cells were maintained in a humidified 37°C, 5%CO_2 incubator, and passaged by partial digestion with 0.25% trypsin and 0.53 mM EDTA in Ca^{2+}-free and Mg^{2+}-free PBS.

Stable transfection of factor inhibiting HIF-1 (FIH)

Plasmid pcDNA3.1/V5-His vector was from Invitrogen. Plasmid pcDNA3.1/V5-His-FIH was described previously [19], and kindly provided by Dr. Bruick at University of Texas Southwestern Medical Center. Cells were transfected with pcDNA3.1/V5-His or pcDNA3.1/V5-His-FIH by Lipofectamine 2000 (Invitrogen). The neomycin-resistant clones were selected, expanded in media containing 500μg/ml G418 (Merck, NJ), and confirmed by assessing both mRNA and protein expressions of FIH.

siRNA of HIF-1α

For RNA interference-mediated knock down of HIF-1α, double-stranded oligonucleotides 5'-ATG ACA TGA AAG CAC AGA T-3' targeting nt 244-262 of human HIF-1α mRNA, which has been confirmed to be effective in down-regulating HIF-1α [20], were annealed and subcloned into BglII- and HindIII-digested pSUPER-Neo vector (OligoEngine, WA). The construct was designated as pSUPER-Neo-Si-HIF-1α A negative pSUPER-Neo-Si-control was constructed by using a 19 nucleotide sequence (5'-ACG CAT GCA TGC TTG CTT T-3') without homology to any mammalian gene sequence [21]. Cells were transfected with pSUPER-Neo-Si-control or pSUPER-Neo-Si-HIF-1α by Lipofectamine 2000 (Invitrogen). The neomycin-resistant clones were selected, expanded in media containing 500μg/ml G418, and confirmed by assessing both mRNA and protein expressions of HIF-1α.

In Vitro hypoxia

To induce *in vitro* hypoxia, monolayers were exposed to reduced oxygen (1% O_2) at 37°C. Briefly, culture plates were placed into a sealed modular incubator chamber (Billups-Rothenberg, CA) purged with 1% O_2, 5% CO_2, and 94% N_2, and then the chamber was maintained at 37°C.

Measurement of transendothelial electrical resistance

Cells were seeded at 1.5×10^5/cm^2 on 0.1% gelatin-coated permeable polycarbonate membrane Transwell supports

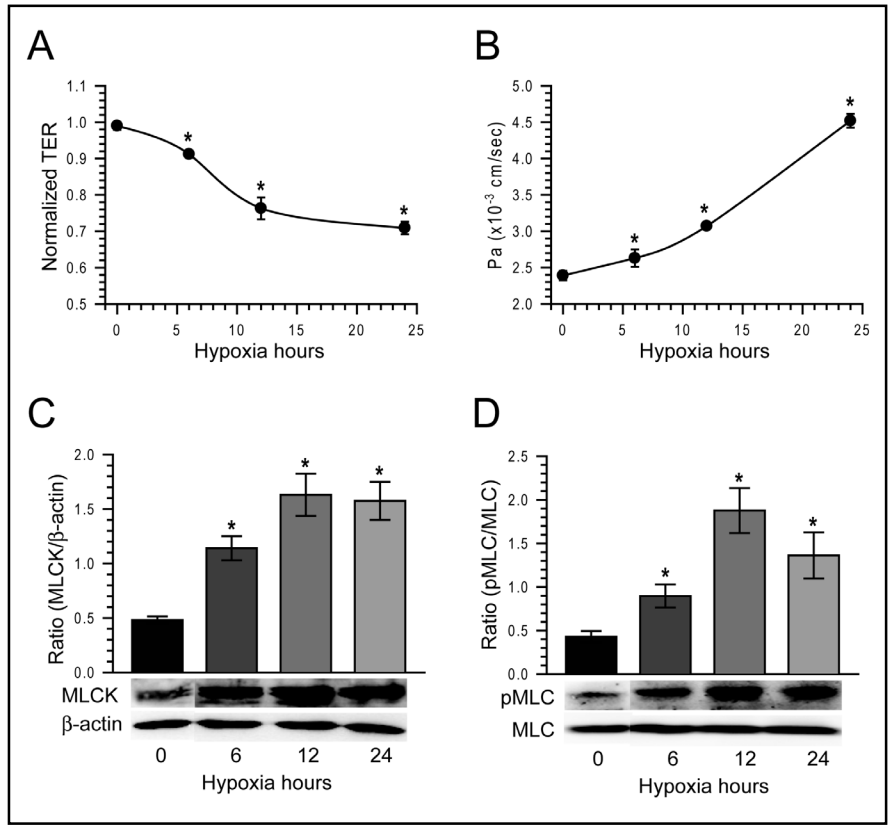


Fig. 1. The hypoxia-induced barrier dysfunction is accompanied by upregulation of both MLCK expression and MLC phosphorylation.A. VE monolayers were exposed to hypoxia for the hours indicated. The TER decreased significantly after hypoxia. TER data normalized to normoxic monolayers represent the mean±SEM (n=6). *, $p<0.05$, compared with 0 hour. B. VE monolayers were incubated as described above. Paracellular permeability was significantly increased in monolayers exposed to hypoxia. The permeability coefficient of albumin (Pa) represents the mean±SEM (n=6). *, $p<0.05$, compared with 0 hour. C. VE monolayers were exposed to hypoxia for the hours indicated. Hypoxia caused a significant increase in MLCK. *, $p<0.05$, compared with 0 hour. n=3. D. Hypoxia caused a significant increase in pMLC, but not MLC. *, $p<0.05$, compared with 0 hour. n=3.

(Corning-Costar, MA) with 0.4μm pores and 6.5mm diameter, and grown to confluence. The monolayers were used 4-5 days after confluence. Transendothelial electrical resistance (TER) was measured using the Milicell ERS volt/ohmmeter (Millipore, MA). TER was calculated by subtracting the resistance value of the filters and fluids, and normalized to that of control monolayers in the same experiment.

Paracellular permeability assay

The luminal-to-abluminal flux of fluorescein isothiocyanate-labeled albumin (FITC-albumin) (Sigma, MO) across the monolayers was assayed as described previously [22], with minor modification. Briefly, monolayers were washed free of media and then transferred to Hanks' balanced salt solution (HBSS). The luminal (top) chamber was gently aspirated and replaced with 100 μl of 1 mg/ml of FITC-albumin and 600 μl HBSS was added to the abluminal (bottom) chamber. The monolayers were incubated at 37°C for 45 min. Fluids from both the luminal and abluminal chambers were sampled. Fluorescence intensity of each sample was measured by fluorospectrophotometry (Hitachi, Japan) with the excitation wave at 485nm and emission wave at 530nm. FITC-albumin concentrations were calculated from standard curves generated by serial dilution of FITC-albumin. The permeability coefficient of albumin (Pa) was then calculated by the previously reported equation [23], i.e. $Pa = [A]/t \times (1/A) \times V/[L]$, where [A] is FITC-albumin concentration, t is time in seconds, A is area of membrane in cm^2, V is volume of abluminal chamber, and [L] is luminal concentration.

Semi-quantitative RT-PCR

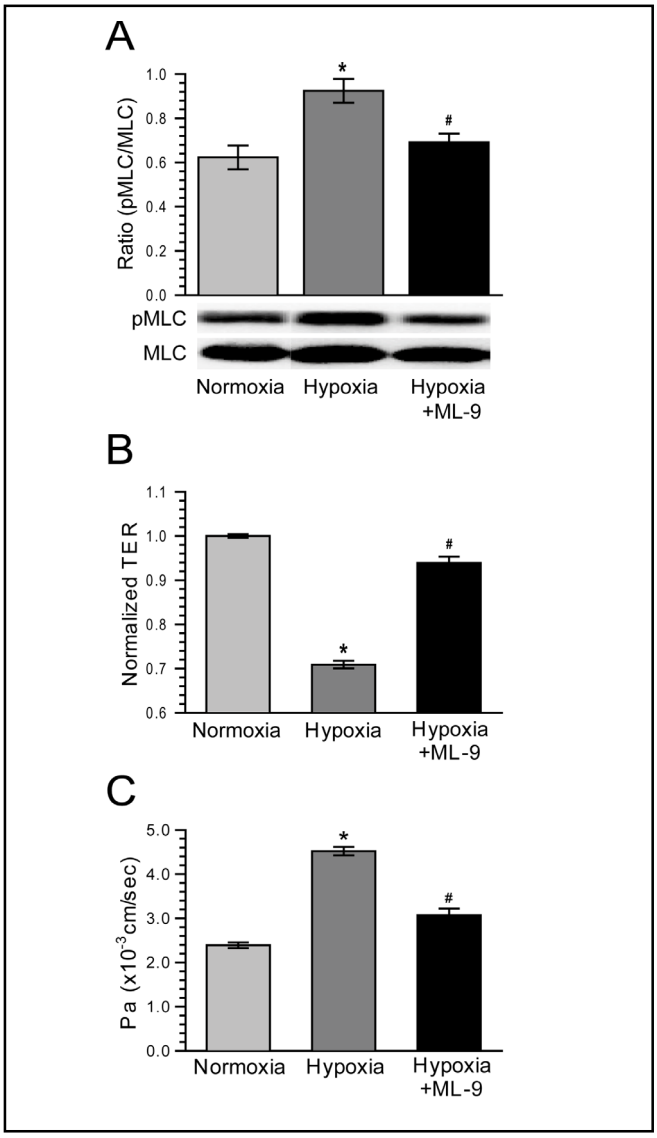
Total RNA purified from TRIzol (Invitrogen) lysates was reversed to cDNA using ThermoScript RT-PCR System (Invitrogen). The reaction was performed in thermocycler (Bio-Rad, CA) at 25°C for 10 min, 50°C for 45 min, and 85°C for 5 min. PCR reaction was performed using ThermoScript RT-PCR System (Invitrogen) according to the default PCR protocol (94°C for 5 min, and 35 cycles of 94°C for 45s, 50°C for 30s, 72°C for 45s, and 72°C for 10min). The primers used in this study were as follows.

GAPDH: 5'-TCA ACG GAT TTG GTC GTC GTA TTG-3'(sense),
5'-TGG AAG ATG GTG ATG GGA TT-3' (antisense);
HIF-1α: 5'-TAA TGT GAG TTC GCA TCT TG -3' (sense),
5'-CAG GTC ATA GGT GGT TTC TT-3'(antisense);
FIH: 5'-CAGTTGCGCAGTTATAGCTTC-3'(sense),
5'-GCC ATC TTC TTC TCA TCA TAG-3'(antisense).

Western immunoblot

Cells were washed with ice-cold PBS, lysed with SDS-PAGE sample buffer containing 50mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol, 10% glycerine. After a brief sonication, samples were centrifuged at 12,000 rpm for 20min at 4°C. The supernatant protein concentrations were determined by RC DC kit (Bio-Rad). The proteins were separated on 10% SDS-PAGE gel followed by transferring to PVDF membrane (Bio-Rad). After blocking, membranes were incubated with antibodies specific for MLCK (Sigma), MLC (Sigma), phosphorylated MLC (Cell Signaling, MA), HIF-1α (Upstate, NY), HIF-1β (Upstate, NY), glucokinase (GK) (Santa Cruz, CA),

Fig. 2. MLCK activity is involved in the hypoxia-induced endothelial barrier dysfunction A. VE monolayers were pretreated with vehicle alone or 100 μmol/L ML-9 for 1 hour followed by exposure to normoxia or hypoxia for 24 hours. Hypoxia induced a significant increase in pMLC, but not MLC. However, pretreatment with ML-9 blocked the MLC phosphorylation induced by hypoxia. The data represent the mean±SEM (n=3). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with hypoxia. B. VE monolayers were treated as described in the legend for panel A. The TER of the monolayers fell significantly after hypoxia. However pretreatment of monolayers with 100 μmol/L ML-9 significantly prevented the TER drop induced by hypoxia. TER data normalized to normoxic monolayers represent the mean±SEM (n=4). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with hypoxia. C. VE monolayers were treated as above. Pretreatment of monolayers with ML-9 significantly prevented the hypoxia-induced increase of paracellular permeability. Pa data represent the mean±SEM (n=4). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with hypoxia.



glucose transporter-1 (GLUT-1) (Santa Cruz), FIH (Novus, CO), and β-actin (Sigma) overnight at 4°C. After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (KPL, MD) for 1 hour. The blots were visualized using Super Signal West Pico (Pierce, IL). The chemiluminescence signal was captured using a ChemiDoc XRS system (Bio-Rad).

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared with Nucbuster Protein Extraction kit (Novagen, WI). The oligonucleotide probe containing HIF-1α binding site (5'-AGC TTG CCCT ACG TGC GTT CTC AGA-3') labeled with biotin was incubated with 10μg of nuclear extract for 20 min at room temperature. The protein-DNA complexes were fractionated on a 5% native polyacrylamide gel and visualized by Light Shift EMSA kit (Pierce) according to manufacturer protocol.

Immunofluorescence

For HIF-1α staining, monolayers were fixed in 1% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After blocking in 5% goat serum for 30 min, monolayers were incubated with rabbit anti-HIF-1α (Upstate, 1:100) overnight at 4°C. Monolayers were washed and incubated with Alexa 594-conjugated goat anti-rabbit antibody (Invitrogen, 1:500) for 60 min. The nuclei were stained with DAPI (Sigma, 1:1000). After extensive washing, monolayers were mounted in Slowfade (Invitrogen). The images were captured using a laser scanning fluorescence microscopy (TCS SP5, Leica, Germany).

Statistical analysis

Results are shown as the means ± SEM. Differences among groups were assessed using analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test. $p<0.05$ was considered statistically significant.

Results

Barrier dysfunction is accompanied by upregulation of both MLCK expression and MLC phosphorylation in endothelial monolayers exposed to hypoxia

It is well documented that hypoxia is capable of reducing endothelial barrier function both *in vivo* and *in vitro* [24-26]. Therefore, our first objective was to determine if hypoxia also induced barrier dysfunction in VE monolayers. As shown in Fig. 1A, when VE monolayers were exposed to hypoxia, the TER, a very sensitive index of endothelial barrier function, decreased significantly. In contrast to the changes in TER, the paracellular permeability of VE monolayers exposed to hypoxia increased progressively (Fig. 1B). These findings

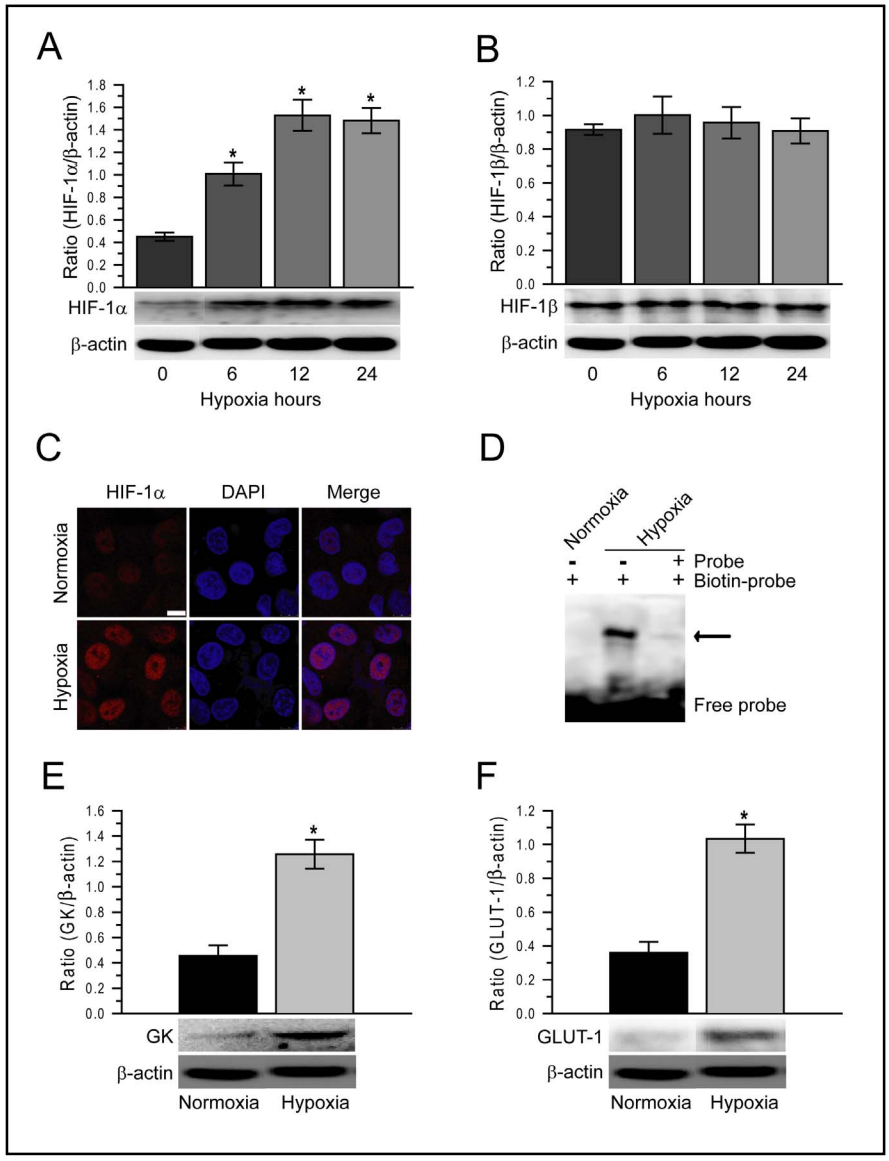


Fig. 3. Hypoxia activates HIF-1 in endothelial cells. A. Hypoxia caused a significant increase in HIF-1α expression. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with 0 hour. B. HIF-1β expression was not significantly afftected by hypoxia exposure. Data represent the mean±SEM (n=3). C. After exposure to normoxia or hypoxia for 6 hours, cells were stained for HIF-1α by immuno-fluorescence. Hypoxia increased the amount of HIF-1α that accumulated within the nucleus. Data are representative of three independent experiments performed in duplicate. Scale bar =10μm. D. Cells were exposed to normoxia or hypoxia for 6 hours. The nuclear extracts were prepared to detect DNA-binding activity of HIF-1 by EMSA. HIF-1 DNA binding was not detected in normoxic cells. However, hypoxia induced the formation of HIF-1 DNA complexes, which were inhibited by the unlabeled specific oligonucleotide probe. Data are representative of three independent experiments performed in duplicate. E. Exposure of cells to hypoxia for 6 hours increased GK expression. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with normoxia. F. Exposure of cells to hypoxia for 6 hours increased GLUT-1 expression. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with normoxia.

were consistent with the drop in TER after exposure to hypoxia. These results strongly indicate that hypoxia is capable of disrupting endothelial barrier function *in vitro*.

Previous studies have shown that MLC phosphorylation plays a critical role in the regulation of endothelial barrier function [27, 28]. Other studies have established that upregulation of MLCK protein expression is pivotal to epithelial barrier dysfunction [22, 29, 30]. Thus we considered the possibility that MLCK protein upregulation and MLC phosphorylation regulated by MLCK are involved in the hypoxia-induced barrier dysfunction in VE monolayers. To test this possibility, we first examined MLCK protein expression in VE cells exposed to hypoxia. As shown in Fig. 1C, MLCK protein expression increased significantly in hypoxic VE cells when compared with that in normoxic VE cells, indicating that the upregulation of MLCK protein is induced by hypoxia. Consistent with this, hypoxic exposure increased the expression of phosphorylated MLC (pMLC) while total MLC expression was not affected (Fig. 1D). The increases of both MLCK protein expression and MLC phosphorylation were in accordance with the changes of both TER and paracellular permeability in endothelial monolayers exposed to hypoxia.

MLCK activity is involved in the hypoxia-induced endothelial barrier dysfunction

Given that the hypoxia-induced endothelial barrier dysfunction was accompanied by upregulation of both MLCK expression and MLC phosphorylation, we next asked whether inhibition of MLCK activity could prevent endothelial barrier dysfunction induced by hypoxia. Our

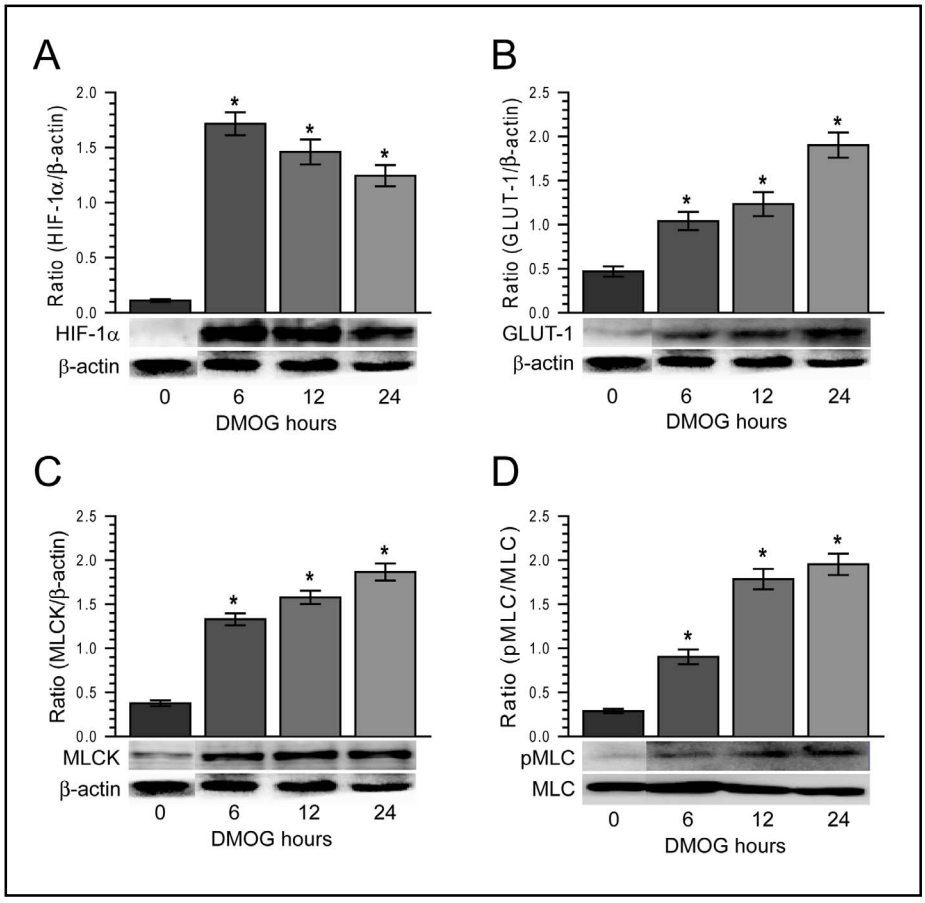


Fig. 4. DMOG-induced HIF-1α activation is accompanied by an increase in MLCK expression and MLC phosphorylation in normoxic cells. A. Normoxic cells were treated with 1mmol/L DMOG for the hours indicated. DMOG significantly increased HIF-1α expression. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with 0 hour. B. Normoxic cells were treated with 1mmol/L DMOG for the hours indicated. DMOG significantly increased GLUT-1 expression. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with 0 hour. C. DMOG treatment induced a significant increase in MLCK expression in normoxic cells. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with 0 hour. D. DMOG treatment induced a significant increase in pMLC, but not MLC in normoxic cells. Data represent the mean±SEM (n=3). *, $p<0.05$, compared with 0 hour.

preliminary experiment proved that ML-9, a specific MLCK inhibitor [31, 32], did not affect barrier function and MLC phosphorylation of VE monolayer in normoxia (data not shown). However, treatment of VE monolayers with ML-9 not only significantly lowered pMLC level in hypoxic VE cells without affecting MLC expression (Fig. 2A), but also effectively prevented both the drop of TER and the increase of paracellular permeability after hypoxic exposure (Fig. 2B, C). These data suggest that MLCK activity and subsequent MLC phosphorylation are involved in endothelial barrier dysfunction induced by hypoxia.

MLCK upregulation is accompanied by HIF-1α activation

A number of laboratories have clearly established that HIF-1 plays a critical role in numerous physiological and pathophysiological responses to hypoxia [8-12]. The data above show that hypoxia induces the upregulation of MLCK protein expression in endothelial cells. Thus we hypothesized that hypoxia-induced upregulation of MLCK might be mediated by HIF-1, an important transcription factor that regulates gene expression in response to cellular hypoxia. We first did a time-course analysis of HIF-1α and HIF-1β protein expression in VE cells exposed to hypoxia. As shown in Fig. 3A, HIF-1α protein expression was significantly increased in hypoxic VE cells, but the expression of HIF-1β protein was not changed significantly (Fig. 3B). Similarly, exposure of VE cells to hypoxia for 6 hours also induced the nuclear accumulation and DNA binding activity of HIF-1α (Fig. 3C, D). Consistent with these, the transcriptional activity of HIF-1α is increased in VE cells exposed to hypoxia (Fig. 3E, F) as supported by the increased expression of GK and GLUT-1, whose genes are targeted by HIF-1α [9, 33, 34]. Taken together, it is clearly suggested that HIF-1α is activated in VE cells under hypoxic conditions.

Specific induction of HIF-1α is capable of increasing MLCK expression and MLC phosphorylation

Given that HIF-1α is activated by hypoxia in VE cells, we considered the possibility that HIF-1α activation is associated with MLCK upregulation in hypoxia. To test this possibility, dimethyloxaloyl glycine (DMOG), a cell permeable HIF-1α inducer which acts to stabilize HIF-1α expression at normal oxygen tensions by competitively

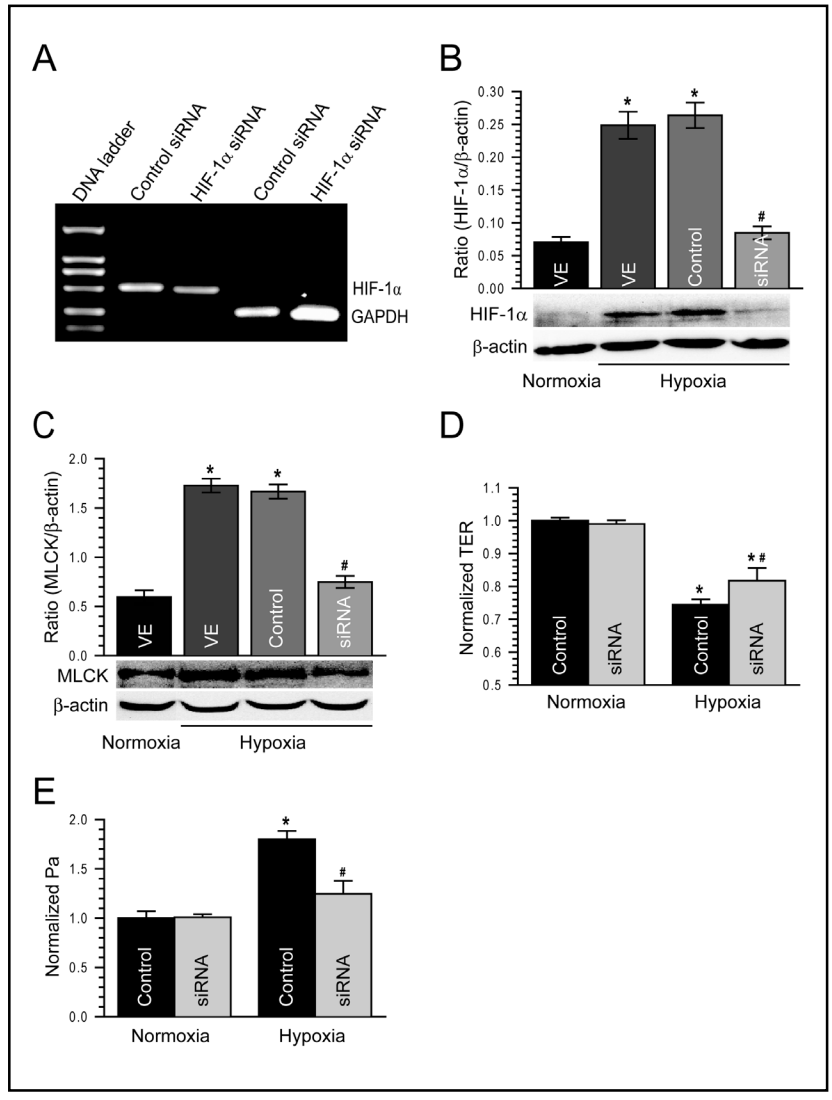


Fig. 5. HIF-1α mediates the endothelial barrier dysfunction and MLCK upregulation induced by hypoxia. A. RT-PCR confirmed that HIF-1α mRNA expression in cells stably transfected with pSUPER-Neo-Si-HIF-1α was effectively inhibited. B. Cells stably transfected with pSUPER-Neo-Si-HIF-1α were exposed to hypoxia for 12 hours. The increase of HIF-1α expression induced by hypoxia was significantly knocked down by stable transfection of pSUPER-Neo-Si-HIF-1α. Data expressed in mean±SEM (n=3). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with VE and control under hypoxic conditions. C. The stable transfectants were exposed to hypoxia for 12 hours. Knockdown of HIF-1α significantly attenuated the increase in MLCK expression induced by hypoxia. Data expressed in mean±SEM (n=3). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with VE and control under hypoxic conditions. D. The stable transfectant monolayers were exposed to hypoxia for 12 hours. The hypoxia-induced decrease in TER was partially reduced by HIF-1α siRNA. TER data normalized to normoxic control represent the mean±SEM (n=6). *, $p<0.05$, compared with normoxic control or HIF-1α siRNA. #, $p<0.05$, compared with hypoxic control. E. Cells were treated as described in the legend for panel C. The hypoxia-induced increase in paracellular permeability was partially prevented by HIF-1α siRNA. Pa data normalized to normoxic control represent the mean±SEM (n=6). *, $p<0.05$, compared with normoxic control. #, $p<0.05$, compared with hypoxic control.

inhibiting the oxygen-sensing enzymes prolyl-4-hydroxylase (PHD), was used to treat VE cells in normoxia. It has well been established that DMOG is capable of inducing HIF-1α expression by preventing HIF-1α degradation in normoxic cells [35, 36]. As shown in Fig. 4A, the HIF-1α expression was significantly increased by treatment of VE cells with 1 mmol/L DMOG in normoxia. In accordance with this, DMOG treatment also significantly increased the expression of HIF-1 target gene GLUT-1 in normoxia (Fig. 4B). These data suggest that DMOG is capable of inducing the expression and transcription activity of HIF-1 in normoxic VE cells. To our surprise, the expression of MLCK and pMLC, but not MLC, was also induced by DMOG treatment of normoxic VE cells (Fig. 4C, D), indicating that the activated HIF-1α is associated with the hypoxia-induced upregulation of MLCK in endothelial cells.

HIF-1α mediates endothelial barrier dysfunction and MLCK upregulation induced by hypoxia

The data above indicate that HIF-1α is associated with MLCK upregulation in hypoxic VE cells. We hypothesized that HIF-1α plays a critical role in the regulation of barrier dysfunction and MLCK expression in hypoxic endothelial cells. To test this hypothesis, VE cells were stably transfected with plasmid pSUPER-si-HIF-1α, which contains shRNA oligonucleotides targeting nt 244-262 of the human HIF-1α mRNA, to knock down HIF-1α in endothelial cells. Stable transfectants were confirmed by analyzing both mRNA and protein levels of HIF-1α under hypoxic condition. HIF-1α mRNA was significantly knocked down in VE cells stably transfected with pSUPER-si-HIF-1α (Fig. 5A). In parallel with this, the HIF-1α protein was also significantly knocked down in pSUPER-si- HIF-1α transfectants (Fig. 5B). These

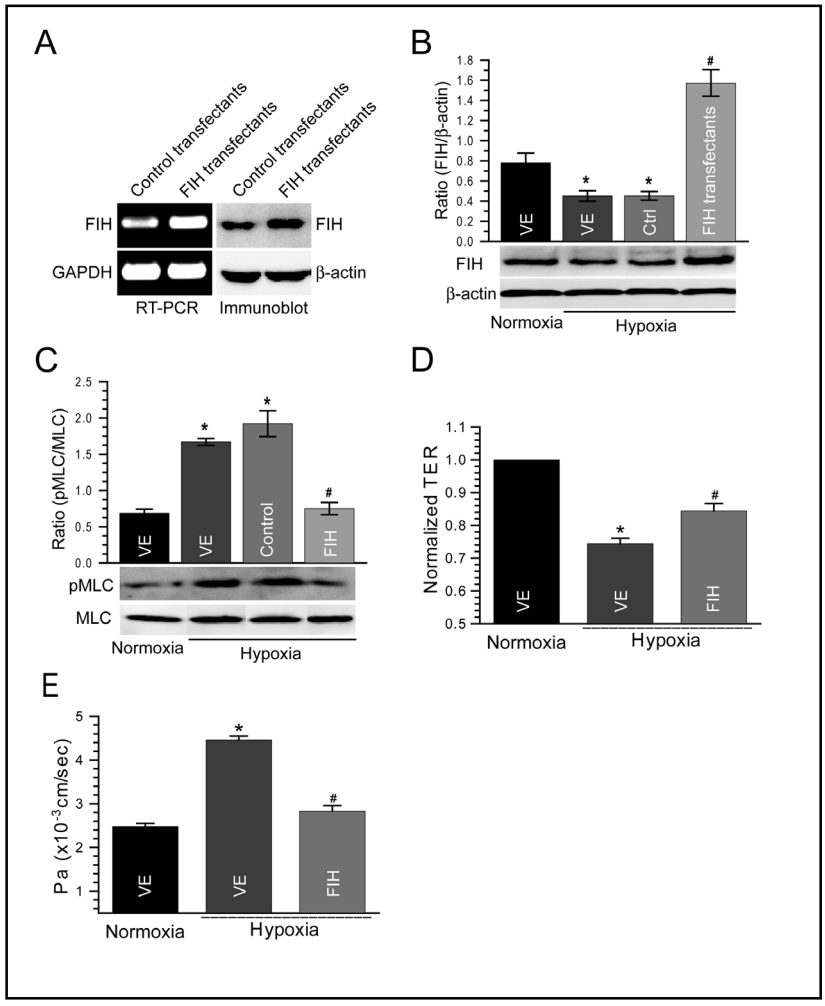


Fig. 6. Inhibition of HIF-1 transcription activity by FIH prevents the hypoxia-induced endothelial barrier dysfunction. A. Plasmid pcDNA3.1/V5-His -FIH was stably transfected into cells. The expression of FIH mRNA and protein in FIH transfectants was significantly increased. Data are representative of three similar experiments, each performed in duplicate.B. FIH expression was significantly increased in hypoxic FIH transfectants. Data expressed in mean±SEM (n=3). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with VE and control. C. Stable overexpression of FIH inhibited the hypoxia-induced increase of pMLC. Data expressed in mean±SEM (n=3). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with VE and control under hypoxic conditions. D. The stable transfectants monolayers were exposed to hypoxia for 12 hours. The hypoxia-induced decrease in TER was partially reduced by FIH overexpression. TER data normalized to normoxic VE control represent the mean±SEM (n=6). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with hypoxic control under hypoxic conditions. E. Cells were treated as described in the legend for panel E. The hypoxia-induced increase in paracellular permeability was partially prevented by FIH overexpression. Pa data represent the mean±SEM (n=6). *, $p<0.05$, compared with normoxia. #, $p<0.05$, compared with hypoxic control under hypoxic conditions.

stable transfectants were then grown as monolayers for assessing MLCK expression and barrier function after hypoxia. As shown in Fig. 5C, knockdown of HIF-1α significantly attenuated hypoxia-induced increase of MLCK expression. Consistent with this, knockdown of HIF-1α also partially prevented the hypoxia-induced drop in TER and the increase in paracellular permeability (Fig. 5D, E). These results indicate that HIF-1α is involved in the MLCK-dependent endothelial barrier dysfunction induced by hypoxia.

Inhibition of HIF-1 transcription activity prevents the hypoxia-induced endothelial barrier dysfunction

Having determined that HIF-1α is involved in hypoxia-induced endothelial barrier dysfunction, we further asked whether FIH, a factor inhibiting the transcription activity of HIF-1 [19, 37-39], was capable of protecting endothelial barrier dysfunction induced by hypoxia. Thus, VE cells were stably transfected with pcDNA3.1/V5-His-FIH, a plasmid containing the full length of human FIH cDNA. The stable transfectants were confirmed by assessing both mRNA and protein expression of FIH. As illustrated in Fig. 6A, both mRNA and protein levels of FIH were significantly increased in the stable transfectants. Similarly, the hypoxia-induced decrease of FIH was also prevented by overexpressing FIH (Fig. 6B). Thus, we further asked if FIH overexpression was capable of preventing the MLC phosphorylation-dependent endothelial barrier dysfunction induced by hypoxia. As shown in Fig. 6C, stable overexpression of FIH significantly prevented the hypoxia-induced increase of pMLC in VE monolayers, whereas the MLC was not affected. In addition, stable overexpression of FIH partially attenuated hypoxia-induced endothelial barrier dysfunction. Both the decrease in TER and increase in paracellular permeability were significantly reduced in hypoxic monolayers stably transfected with FIH. (Fig. 6D, E). These data suggest that FIH partially prevents hypoxia-induced barrier

dysfunction by inhibiting MLC phosphorylation in endothelial cells.

Discussion

It is known that endothelial cells, which line the inner lumen of blood vessels and form a semi-permeable dynamic barrier between the blood vessels and underlying tissues, are the primary determinants of vascular permeability. Under some pathophysiological conditions, loss of endothelial barrier function is predominantly due to an increase in paracellular permeability, leading to extravasation of macromolecules and fluid. Though early *in vitro* study has demonstrated that hypoxia does not affect intestinal barrier function directly [40], hypoxia has recently been reported to significantly increase intestinal permeability of mice exposed to hypoxia [41]. Similarly, previous studies have shown that hypoxia induces endothelial barrier dysfunction both *in vivo* and *in vitro* [24-26, 42, 43]. Consistent with those studies, our present data also demonstrate that hypoxia is capable of inducing vascular endothelial barrier dysfunction, as reflected by the decreased TER and increased paracellular permeability.

A key finding in this study is that hypoxia-induced endothelial barrier dysfunction is dependent upon the upregulation of MLCK protein expression. Although hypoxia causes endothelial barrier dysfunction, the intracellular mechanisms are not well characterized. Previous studies have suggested that hypoxia increases endothelial permeability via the release of VEGF, which subsequently leads to dislocalization, decreased expression, and enhanced phosphorylation of the TJ protein ZO-1 [16, 17]. Similarly, a recent study showed that hypoxia disrupts endothelial barrier function by reducing expression of the TJ protein claudin-5 [44]. However, MLC phosphorylation is considered to be a central signaling event leading to increased TJ permeability. For example, MLC phosphorylation is involved in contractile events, gap formation and barrier dysfunction of endothelial cells [27, 28]. MLC phosphorylation alone is sufficient to increase TJ permeability, which is associated with biological and morphological reorganization of the TJ proteins ZO-1 and occludin [45]. Our current data show that hypoxia-induced endothelial barrier dysfunction is accompanied by increased MLC phosphorylation. Inhibiting the activity of MLCK prevented both the barrier dysfunction and MLC phosphorylation caused by hypoxia, suggesting that MLCK-dependent MLC phosphorylation is involved in the mechanisms by which hypoxia disrupts endothelial barrier function.

It has been established that MLCK is primarily responsible for MLC phosphorylation. We [22, 29] and other investigators [30] have demonstrated that upregulation of MLCK protein expression is critical to intestinal epithelial barrier dysfunction induced by cytokines. The implication of these studies is not limited to intestinal epithelial barrier dysfunction because MLCK is expressed ubiquitously and regulates diverse cellular functions. Thus, we asked whether increased MLC phosphorylation is accompanied by an upregulation of MLCK protein expression in hypoxic endothelial cells. The present data show that MLCK protein expression was significantly increased by hypoxia in endothelial cells. These data suggest that MLCK protein upregulation is associated with barrier dysfunction in hypoxic endothelial monolayers.

The most striking finding in this study is that HIF-1α regulates the MLCK-dependent barrier dysfunction induced by hypoxia in endothelial cells. Having found that MLCK protein expression is upregulated by hypoxia, we hypothesized that HIF-1α regulates endothelial barrier dysfunction in hypoxia by inducing MLCK expression since HIF-1α is principally responsible for causing the hypoxia response. We first assessed HIF-1 activation in endothelial cells exposed to hypoxia. Our data show that HIF-1 is activated in hypoxic endothelial cells, as reflected not only by the increased HIF-1α expression, nuclear accumulation, and DNA binding activity of HIF-1, but also by the upregulated expression of GK and GLUT-1, two HIF-1 target genes. We next considered whether activation of HIF-1α is associated with the upregulation of MLCK protein expression and increased MLC phosphorylation in hypoxic endothelial cells. The data show that even in normoxic endothelial cells, DMOG, which upregulates HIF-1α expression by inhibiting the activity of PHD [35, 36], not only induces HIF-1α activity, but also leads to the upregulation of MLCK protein and MLC phosphorylation. Taken together, it is suggested that HIF-1α activation by PHD inhibition is associated with MLCK upregulation and MLC phosphorylation in hypoxic endothelial cells, and that PHD, a vital negative regulator of HIF-1α might be involved in the hypoxia-induced endothelial barrier dysfunction via a MLCK-dependent pathway.

To confirm the role of HIF-1α in regulating MLCK-dependent barrier dysfunction in hypoxic endothelial cells, we investigated MLCK expression and barrier function in endothelial monolayers with HIF-1α knockdown. The

data demonstrate that knockdown of HIF-1α significantly attenuates the increased MLCK expression induced by hypoxia. Consistent with this, knockdown of HIF-1α also partially attenuates the endothelial barrier dysfunction caused by hypoxia. These data suggest that while HIF-1α is involved in the MLCK-dependent endothelial barrier dysfunction induced by hypoxia, it is not the unique mediator. Similarly, our data also show that FIH overexpression inhibits the hypoxia-induced increase in transcription activity of HIF-1 and MLC phosphorylation, with a partial reduction in barrier dysfunction. This indicates that inhibition of HIF-1 transcription activity can partially prevent the endothelial barrier dysfunction caused by hypoxia, and other mediators and pathways should be considered in the pathogenesis of endothelial barrier dysfunction in hypoxia. The mechanism by which HIF-1α regulates MLCK pathway is currently not clear. We [46] and others [47] have previously shown that NF-κB are involved in the transcriptional upregulation of MLCK in intestinal epithelial cells exposed to cytokines. It has been previously reported that HIF-1α itself contributes to NF-κB activation. For example, NF-κB activation is diminished in HIF-1α-deficient murine neutrophils [48], and both p65 nuclear localization and transcriptional activity are induced in mice with increased epithelial levels of HIF-1α [49]. Thus, HIF-1α probably induces MLCK expression by enhancing NF-κB activation. But this expected mechanism needs expanded studies. Consistent with our finding, recent studies also implicate that HIF-1α is critical to the barrier dysfunction associated with hypoxia. For example, inhibiting HIF-1α with YC-1, a specific antagonist of HIF-1α prevents the endothelial barrier dysfunction and ZO-1 disorganization caused by hypoxia [18]. An *in vivo* study demonstrated that mice conditionally lacking intestinal epithelial *hif1α* are resistant to increased intestinal epithelial permeability induced by hypoxia [41]. It has also reported that partial HIF-1α deficiency attenuates gut and lung injury induced by ischemia/reperfusion injury, and persistent HIF-1α activation is linked to loss of gut barrier function [50, 51]. Furthermore, HIF-1α augments experimental murine colitis induced by dextran sulfate sodium [52]. Thus, these studies indicate that HIF-1α is injurious to epithelial barrier function. In contrast, it has been reported that HIF-1α decreases vascular permeability[53]. Some studies have also shown that HIF-1α is protective to intestinal barrier function in experimental murine colitis [54, 55]. Hence, the role of HIF-1α in barrier function regulation is still controversial. Although future studies are necessary to specifically define the precise mechanism by which HIF-1α regulates barrier function, a regulatory role for HIF-1α in the MLCK-dependent endothelial barrier dysfunction induced by hypoxia should be considered.

In summary, the data presented here demonstrate that the MLCK-dependent MLC phosphorylation pathway contributes to the hypoxia-induced endothelial barrier dysfunction. The MLCK-dependent endothelial barrier dysfunction induced by hypoxia is associated with the activation of HIF-1α. Our data have, for the first time, suggested that HIF-1α regulates hypoxia-induced endothelial barrier dysfunction by upregulating MLCK protein expression and subsequent MLC phosphorylation. Thus, our current studies provide a new insight into the mechanisms of the hypoxia-induced endothelial barrier dysfunction.

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References

1 Mullin JM, Agostino N, Rendon-Huerta E, Thornton JJ: Keynote review: epithelial and endothelial barriers in human disease. Drug Discov Today 2005;10:395-408.

2 Schneeberger EE, Lynch RD: The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 2004;286:C1213-C1228.

3 Mehta D, Malik AB: Signaling mechanisms regulating endothelial permeability. Physiol Rev 2006;86:279-367.

4 Orlova VV, Economopoulou M, Lupu F, Santoso S, Chavakis T: Junctional adhesion molecule-C regulates vascular endothelial permeability by modulating VE-cadherin-mediated cell-cell contacts. J Exp Med 2006;203:2703-2714.

5 van Hinsbergh V, van Nieuw Amerongen G: Intracellular signaling involved in modulating human endothelial barrier function. J Anat 2002;200:549-560.

6 Harhaj NS, Antonetti DA: Regulation of tight junctions and loss of barrier function in pathophysiology. Int J Biochem Cell Biol 2004;36:1206-1237.

7 Semenza GL: Life with oxygen. Science 2007;318:62-64.
8 Cummins EP, Taylor CT: Hypoxia-responsive transcription factors. Pflugers Arch 2005;450:363-371.
9 Semenza GL: HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000;88:1474-1480.
10 Semenza GL: Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Ann Rev Cell Dev Biol 1999;15:551-578.
11 Michiels C, Minet E, Michel G, Mottet D, Piret JP, Raes M: HIF-1 and AP-1 cooperate to increase gene expression in hypoxia: role of MAP kinases. IUBMB Life 2001;52:49-53.
12 Michiels C, Minet E, Mottet D, Raes M: Regulation of gene expression by oxygen: NF-kappaB and HIF-1, two extremes. Free Radic Biol Med 2002;33:1231-1242.
13 Semenza GL: HIF-1 and human disease: one highly involved factor. Genes Dev 2000;14:1983-1991.
14 Semenza GL: HIF-1 and mechanisms of hypoxia sensing. Curr Opin Cell Biol 2001;13:167-171.
15 Ratcliffe PJ, O'Rourke JF, Maxwell PH, Pugh CW: Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol 1998;201:1153-1162.
16 Fischer S, Clauss M, Wiesnet M, Renz D, Schaper W, Karliczek GF: Hypoxia induces permeability in brain microvessel endothelial cells via VEGF and NO. Am J Physiol 1999;276:C812-C820.
17 Fischer S, Wobben M, Marti HH, Renz D, Schaper W: Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. Microvasc Res 2002;63:70-80.
18 Yeh WL, Lu DY, Lin CJ, Liou HC, Fu WM: Inhibition of hypoxia-induced increase of blood-brain barrier permeability by YC-1 through the antagonism of HIF-1α accumulation and VEGF expression. Mol Pharmacol 2007;72:440-449.
19 Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Burick RK: FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 2002;16:1466-1471.
20 Zhang X, Kon T, Wang H, Li F, Huang Q, Rabbani ZN, Kirkpatrick JP, Vujaskovic Z, Dewhirst MW, Li CY: Enhancement of hypoxia-induced tumor cell death in vitro and radiation therapy in vivo by use of small interfering RNA targeted to hypoxia-inducible factor-1α. Cancer Res 2004;64:8139-8142.
21 Baek JH, Mahon PC, Oh J, Kelly B, Krishnamachary B, Pearson M, Chan DA, Giaccia AJ, Semenza GL: OS-9 interacts with hypoxia-inducible factor 1α and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1α. Mol Cell 2005;17:503-512.
22 Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR: Interferon-γ and tumor necrosis factor-α synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol 2005;166:409-419.
23 Yuan SY, Wu MH, Ustinova EE, Guo M, Tinsley JH, de Lanerolle P, Xu W: Myosin light chain phosphorylation in neutrophil-stimulated coronary microvascular leakage. Circ Res 2002;90:1214-1221.
24 Ali MH, Schlidt SA, Hynes KL, Marcus BC, Gewertz BL: Prolonged hypoxia alters endothelial barrier function. Surgery 1998;124:491-497.
25 St Cyr JA, Shecterle LM: Effects of hypoxia on the pulmonary microvascular permeability in adult rabbit lung. J Biomed Sci 1995;2:70-76.
26 Schoch HJ, Fischer S, Marti HH: Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain. Brain 2002;125:2549-2557.
27 Garcia JGN, Schaphorst KL: Regulation of endothelial cell gap formation and paracellular permeability. J Investig Med 1995;43:117-126.
28 Garcia JGN, Davis HW, Patterson CE: Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. J Cell Physiol 1995;163:510-522.
29 Wang F, Schwarz BT, Graham WV, Wang Y, Su L, Clayburgh DR, Abraham C, Turner JR: IFN-γ-induced TNFR2 up-regulation is required for TNF-dependent intestinal epithelial barrier dysfunction. Gastroenterology 2006;131:1153-1163.
30 Ma TY, Boivin MA, Ye D, Pedram A, Said HM: Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. Am J Physiol Gastrointest Liver Physiol 2005;288:G422-G430.
31 Tinsley JH, De Lanerolle P, Wilson E, Ma W, Yuan SY: Myosin light chain kinase transference induces myosin light chain activation and endothelial hyperpermeability. Am J Physiol Cell Physiol 2000;279:C1285-C1289.
32 Connell LE, Helfman DM: Myosin light chain kinase plays a role in the regulation of epithelial cell survival. J Cell Sci 2006;119:2269-2281.
33 Roth U, Curth K, Unterman TG, Kietzmann T: The transcription factors HIF-1 and HNF-4 and the coactivator p300 are involved in insulin-regulated glucokinase gene expression via the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 2004;279:2623-2631.
34 Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL: Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. Genes Dev 1998;12:149-162.
35 Asikainen TM, Schneider BK, Waleh NS, Clyman RI, Ho W, Flippin LA, Günzler V, White CW: Activation of hypoxia-inducible factors in hyperoxia through prolyl 4-hydroxylase blockade in cells and explants of primate lung. Proc Natl Acad Sci USA 2005;102:10212-10217.
36 Jaakkola P, Mole DR, Tian Y, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim AV, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ: Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 2001;292:468-472.
37 Mahon PC, Hirota K, Semenza GL: FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 2001;15:2675-2686.
38 Lancaster DE, McDonough MA, Schofield CJ: Factor inhibiting hypoxia-inducible factor (FIH) and other asparaginyl hydroxylases. Biochem Soc Trans 2004;32:943-945.
39 Kaelin WG: Proline hydroxylation and gene expression. Annu Rev Biochem 2005;74:115-128.
40 Taylor CT, Dzus AL, Colgan SP: Autocrine regulation of epithelial permeability by hypoxia: role for polarized release of tumor necrosis factor alpha. Gastroenterology 1998;114:657-668.
41 Rosenberger P, Khoury J, Kong T, Weissmuller T, Robinson AM, Colgan SP: Identification of vasodilator-stimulated phosphoprotein (VASP) as an HIF-regulated tissue permeability factor during hypoxia. FASEB J 2007;20:2613-2621.
42 Kuhlmann CR, Tamaki R, Gamerdinger M, Lessmann V, Behl C, Kempski OS, Luhmann HJ: Inhibition of the myosin light chain kinase prevents hypoxia-induced blood-brain barrier disruption. J Neurochem 2007;102:501-507.
43 Kimura K, Teranishi S, Kawamoto K, Nishida T: Protection of human corneal epithelial cells from hypoxia-induced disruption of barrier function by hepatocyte growth factor. Exp Eye Res 2010;90:337-343.

44 Koto T, Takubo K, Ishida S, Shinoda H, Inoue M, Tsubota K, Okada Y, Ikeda E: Hypoxia disrupts the barrier function of neural blood vessels through changes in the expression of claudin-5 in endothelial cells. Am J Pathol 2007;170:1389-1397.
45 Shen L, Black ED, Witkowski ED, Lencer WI, Guerriero V, Schneeberger EE, Turner JR: Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. J Cell Sci 2006;119:2095-2106.
46 Graham WV, Wang FJ, Clayburgh DR, Cheng JX, Yoon B, Wang Y, Lin A, Turner JR: Tumor necrosis factor-induced long myosin light chain kinase transcription is regulated by differentiation-dependent signaling events: characterization of the human long myosin light chain kinase promoter. J Biol Chem 2006;281:26205-26215.
47 Ye D, Ma TY: Cellular and molecular mechanisms that mediate basal and tumor necrosis factor-α induced regulation of myosin light chain kinase gene activity. J Cell Mol Med 2008;12:1331-1346.
48 Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N, Chilvers ER: Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. J Exp Med 2005;201:105-115.
49 Scortegagna M, Cataisson C, Martin RJ, Hicklin DJ, Schreiber RD, Yuspa SH, Arbeit JM: HIF-1α regulates epithelial inflammation by cell autonomous NF-κB activation and paracrine stromal remodeling. Blood 2008;111:3343-3354.
50 Feinman R, Deitch EA, Watkins AC, Abungu B, Colorado I, Kannan KB, Sheth SU, Caputo FJ, Lu Q, Ramanathan M, Attan S, Badami CD, Doucet D, Barlos D, Bosch-Marce M, Semenza GL, Xu DZ: HIF-1 mediates pathogenic inflammatory responses to intestinal ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 2010;299:G833-843.
51 Kannan KB, Colorado I, Reino D, Palange DC, Lu Q, Qin X, Abungu B, Watkins AC, Caputo FJ, Xu DZ, Semenza GL, Deitch EA, Feinman R: Hypoxia-inducible factor plays a gut-injurious role in intestinal ischemia reperfusion injury. Am J Physiol Gastrointest Liver Physiol 2010, in press.
52 Shah YM, Ito S, Morimura K, Chen C, Yim SH, Haase VH, Gonzalez FJ: Hypoxia-inducible factor augments experimental colitis through an MIF-dependent inflammatory signaling cascade. Gastroenterology 2008;134:2036-2048.
53 Morote-Garcia JC, Rosenberger P, Kuhlicke J, Eltzschig HK: HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. Blood 2008;111:5571-5580.
54 Robinson A, Keely S, Karhausen J, Gerich ME, Furuta GT, Colgan SP: Mucosal protection by hypoxia-inducible factor prolyl hydroxylase inhibition. Gastroenterology 2008;134:145-155.
55 Cummins EP, Seeballuck F, Keely SJ, Mangan NE, Callanan JJ, Fallon PG, Taylor CT: The hydroxylase inhibitor dimethyloxalylglycine is protective in a murine model of colitis. Gastroenterology 2008;134:156-165.